Generation of Epstein-Barr Virus (EBV)–Immortalized B Cell Lines

BASIC PROTOCOL

Immortalization of B lymphocytes by EBV is an effective procedure for inducing long-term growth of certain human B lymphocytes. The basic protocol described below to accomplish this can be divided into three stages: preparation of virus, preparation of target cells to be immortalized, and EBV infection and growth of infected cells.

CAUTION: EBV may cause disease in nonimmune individuals. Only individuals with serum antibodies to EBV should handle cell lines infected with the virus. In addition, EBV has been associated with a number of lymphoproliferative disorders. Biosafety practices must be followed (see Chapter 7 introduction)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique must be used accordingly.

Materials

Complete RPMI-10 medium (*APPENDIX 2*), without and with 1 µg/ml cyclosporin A (written request from Sandoz; prepare 1 mg/ml stock in 100% ethanol and store at –20°C)
B95-8 cells (marmoset cell line; ATCC #~~CRL 1612~~ VR-1492)
Heparinized peripheral blood (*APPENDIX 3*)
Phosphate-buffered saline (PBS; *APPENDIX 2*)
Hanks balanced salt solution (HBSS; *APPENDIX 2*)

Beckman centrifuge with JS 4.2 rotor (or equivalent)
0.45-µm filter
50-ml conical tubes
25-cm^2 tissue culture flasks

Additional reagents and equipment for cell counting, cryopreservation, and determination of viability (*APPENDIX 3*) and Ficoll-Hypaque gradient centrifugation (*UNIT 7.1*)

Prepare EBV-containing culture supernatants

1. Inoculate complete RPMI-10 with 1×10^6 cells/ml exponentially growing B95-8 cells. Incubate 3 days in a humidified 37°C, 5% CO_2 incubator.

   Cells should be >90% viable.

2. Centrifuge 10 min at 1200 rpm (300 × *g*), 4°C, to separate the EBV-containing culture supernatant from the cells. Filter the supernatant through a 0.45-µm filter, aliquot, and store at –130°C.

   The culture supernatant contains >10^2 to 10^3 transforming units/ml. For determination of EBV titer, see Miller and Lipman, 1973. The virus can be stored >1 year at –130°C without appreciable loss in titer.

Prepare target cells to be immortalized

3. Dilute >15 ml heparinized peripheral blood 1:2 in PBS.

   If tonsil or spleen are used, fragment them into single cell suspensions and adjust to 5×10^6 to 10^7 cells/ml in complete RPMI-10 (UNIT 7.8). If bone marrow is used, dilute 1:20 in complete RPMI-10.

4. Underlay 12 ml diluted blood (or cell suspensions from tonsil, spleen, or bone marrow) with 12 ml Ficoll-Hypaque in a 50-ml conical tube. Centrifuge 8 min at 3000 rpm (1500 × *g*), room temperature, remove buffy coat interface, and transfer to a new 50-ml conical tube.

5. Fill tube with PBS, centrifuge 15 min at 1200 rpm (300 × *g*), room temperature, and discard supernatant.

6. Resuspend cell pellet in HBSS, centrifuge 10 min at 1200 rpm (300 × *g*), room temperature, and discard supernatant. Repeat once.

7. Resuspend cell pellet in 2 to 5 ml complete RPMI-10 and count cells.

   On microscopic examination, mononuclear cell suspensions contain nucleated cells of varying size and shape, including lymphocytes and monocytes.

Infect B cells and culture infected cells

8. Place 10^7 mononuclear cells in 2.5 ml complete RPMI-10 into a 50-ml conical tube. Add 2.5 ml culture supernatant from step 2. Incubate 2 hr in a 37°C water bath.

9. Add 5 ml complete RPMI-10 containing 1 µg/ml cyclosporin A. Transfer the 10-ml cell suspension to a 25-cm^2 tissue culture flask. Stand flask 3 weeks in a humidified 37°C, 5% CO_2 incubator.

   Toward the end of the 3-week incubation, the culture medium becomes acidic and the cells form macroscopic clumps. By phase-contrast microscopy, many cells appear large, clear, often hairy, and tend to form tight clumps of varying size. All these features indicate the occurrence of B cell immortalization by EBV.

10. Mix cells (after 3 weeks) and transfer 5 ml to two new 25-cm^2 tissue culture flasks. Add 5 ml complete RPMI-10 to each flask and incubate 1 week in a humidified 37°C, 5% CO_2 incubator. At this time, the cell line can be cryopreserved at −130°C or maintained in long-term culture.

11. Maintain cell line by splitting 1:3 in complete RPMI-10 once a week. Incubate in a humidified 37°C, 5% CO_2 incubator.

COMMENTARY

Background Information

Epstein-Barr virus is the only virus known to immortalize human B lymphocytes. Only a portion of the circulating B cells (~1 in 100) are immortalized by EBV (Sugden and Mark, 1977), and resting B lymphocytes are immortalized in preference to activated B lymphocytes (Aman et al., 1984). T cells from EBV-seropositive individuals suppress B cell immortalization by EBV in culture (Rickinson et al., 1979). Therefore, immortalization by EBV occurs with greater frequency if the immune T cells are either physically removed from culture or are functionally inactivated (e.g., with cyclosporin A; Tosato et al., 1982).

EBV-immortalized B cell lines are initially polyclonal and secrete all major classes of immunoglobulin. After prolonged culture in vitro, EBV-immortalized cell lines become oligoclonal or monoclonal, reflecting the outgrowth of selected cell clones (Nilsson and Klein, 1982). Typically, EBV-immortalized B cell lines are infected latently with EBV and produce little or no infectious viral particles (Sugden et al., 1979).

Critical Parameters

An adequate number of infectious EBV particles should be added to the cells to be immortalized. While culture supernatants of the virus producer B95-8 cell line usually contain >10^2 to 10^3 transforming units/ml, occasionally the cell line produces little or no virus. Thus, the EBV-containing supernatants should be titrated for their transforming potency before use.

The target B cells for immortalization should be viable, of sufficient number, and resting. Because most adults are EBV-seropositive and have regulatory T cells that prevent growth of autologous EBV-infected B cells, cyclosporin A (a drug that functionally inactivates these cells) is added to the cultures.

Growth of EBV-infected B cells is favored by monocytes and B cell growth factor(s) produced by EBV-infected B cells. Therefore, monocytes should not be removed from mononuclear cells, B95-8 culture supernatant containing EBV should not be removed after B cell infection, culture supernatant should

not be changed during the initial 3-week period of incubation, and the cell line should always be expanded conservatively.

Anticipated Results

This procedure produces a long-term EBV-infected cell line of B cell phenotype, initially secreting polyclonal immunoglobulin. After prolonged culture, the cell line may become oligoclonal or monoclonal.

Time Considerations

Preparation of the EBV-containing culture supernatants takes 3 days. Preparation of the mononuclear cells to be immortalized takes 2 to 3 hr and virus infection takes 2 hr. Generation of the long-term cell line takes 3 to 5 weeks.

Literature Cited

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Key Reference

Nilsson, K. and Klein, G. 1982. See above.

Presents a detailed description of human B lymphoid cell lines.

Contributed by Giovanna Tosato
Center for Biologics Evaluation and Research
Food and Drug Administration
Bethesda, Maryland